Effects of polyphosphates and fluoride on hydroxyapatite dissolution: a pH-stat investigation

Abstract

Objectives: This study investigated the immediate and sustained effect of sodium trimetaphosphate (TMP) and sodium hexametaphosphate (HMP) associated or not with fluoride (F) on hydroxyapatite (HA) dissolution using an erosion-like model, considering the salivary coating formation.

Design: Baseline dissolution rates were determined for HA discs using a pH-stat system. In the first set of experiments, HA discs were treated with 1100 µg F/mL, 1% or 8% of HMP, 1% or 8% of TMP and 1100 µg F/mL associated with 1% or 8% of HMP or TMP, totaling 9 groups (n=8). In a second phase, HA discs were kept in pooled human saliva at 37º C for 2h before treatment with deionised water and 1100 µg F/mL associated with 1% or 8% of HMP or TMP, totaling 5 groups (n=8). The post-treatment dissolution rate was determined from three consecutive 30-min assays. Data were analysed using 2 and 3-way ANOVA followed by Fisher and Holm-Sidak methods, respectively (α=0.05).

Results: All test solutions promoted reduction in HA dissolution rate when compared to baseline control in the first post-treatment run ($p<0.001$). However, a synergistic effect was only observed between fluoride and 1% HMP. Moreover, the duration of inhibitory effect was greater when 8% HMP and 1 or 8% HMP associated with F were assessed ($p<0.001$). The presence of salivary coating led to higher protection for all groups when compared to discs without coating ($p<0.001$).

Conclusion: The reduction of HA dissolution rate, as well as the duration of this effect were influenced by fluoride, type and concentration of phosphate salt and the presence of a salivary coating.

Keywords: erosion; fluoride; polyphosphates; salivary coating; hydroxyapatite.

Introduction

Dental erosion has been recognized as an increasing dental problem among children, adolescents and adults.[1] Thus, there is a growing interest in the development and evaluation of treatments which can reduce the severity and offer protection against dental erosion. In this context, fluoride (F) has been widely used as a complementary preventive measure with the aim of reducing mineral loss.[2] However, in order to design more effective formulations, several active ingredients other than fluorides, or in addition to, have been studied. [3-9]

Promising results have led to an increasing interest in polyphosphates with and without fluoride,[10-13] among these, sodium trimetaphosphate (TMP) and sodium hexametaphosphate (HMP) have been shown to have protective effects in both caries and erosion.[4,5,8,10-17] TMP is a cyclic condensed phosphate and, according to the literature, it would preserve the stability and integrity of the enamel mineral surface during enamel erosion.[18] Sodium hexametaphosphate (HMP) is a cyclic phosphate which has the ability to reduce enamel solubility.[19-21] For both TMP and HMP, an appropriate ratio of phosphate/F should be maintained to achieve favorable effects; it seems to be related to a formation of a "barrier" on the enamel surface which can provide protection against mineral loss in cariogenic and erosive challenges.[8,22]

Considering that the mechanisms of action of TMP and HMP are still not fully elucidated, the assessment of their effects alone or in combination with fluoride would be instructive, especially regarding the interactions of these with the tooth mineral. In this sense, a pH stat system can be used to evaluate the immediate and sustained effect of therapeutic agents on the dissolution of hydroxyapatite discs. This model has been used in previous studies [23-25] as a model for dental tissues in studies of other inhibitors and have been shown to react to solution factors such as pH in a qualitatively similar way as enamel.[26]

Based on the above, the purpose of the present study was to evaluate the demineralisation-inhibiting properties of these phosphates alone or associated with fluoride. Considering that saliva may have a strong influence on dental erosion and on tests of anti-erosive agents, [25,27] the effects of TMP and HMP on dissolution of both native surfaces and of surfaces previously coated with salivary coating were assessed. The study hypothesis was that HA dissolution would be significantly reduced by the presence of fluoride associated or not with TMP or HMP, and that this effect would be enhanced by the presence of salivary coating.

Material and Methods

Hydroxyapatite discs and solutions preparation

Discs of compressed hydroxyapatite (HA) (mean diameter 12.7 mm and thickness 1.39 mm) were acquired from HiMed Inc., Old Bethpage, N.Y., USA. Prior to use in the pH-stat, discs were exposed to gently stirred 0.3% citric acid, pH 3.2, for 30 min at room temperature,

washed in deionised water and finally air-dried to ensure consistency of response and remove loose HA particles. Before use in pH-stat, each disc was coated with nail varnish on the underside to leave a constant exposed area (126.6 mm^2) available for reaction using an established procedure.[24] Then, discs were fixed with sticky wax to the tip of a glass tube to be inserted into the inlet port in a reproducible position.

Solutions were prepared using deionised water and reagents were acquired from Sigma-Aldrich (Poole, Dorset, UK). Solutions of citric acid were prepared at 0.3% concentrations with pH value of 3.20 (adjusted using NaOH). Fluoride (NaF; Poole, Dorset, UK) and phosphates (sodium trimetaphosphate – TMP and sodium hexametaphosphate – HMP; Poole, Dorset, UK) solutions were prepared in the following concentrations: 1100 µg F/mL, 1% and 8% of HMP or TMP and 1100 µg F/mL associated with 1% and 8% of HMP or TMP.

Measurement of Dissolution Rate

A pH-stat system (718 Stat Titrino: Metrohm UK, Runcorn, Cheshire, UK) with a 50-mL double-walled glass reaction vessel and a lid with 3 inlet ports was used to determine the dissolution rate of hydroxyapatite. The reaction temperature was 37 °C maintained using a water-jacketed reaction vessel and a water bath (Type GD120; Grant Instruments, Cambridge, UK). For each dissolution measurement, 30 ml of 0.3% citric acid solution was introduced into the reaction vessel and pH electrode and burette tip adapted. After the system had reached thermal equilibrium, the pH was adjusted to 3.2 by adding 1M NaOH and then performing final fine adjustment using the pH-stat. The reaction was initiated by immersing the HA disc into the reaction vessel containing the citric acid gently stirred and addition of titrant (50 mM HCl) was recorded for 30 min. A baseline measurement of dissolution rate was determined for each disc from the mean of three 30-min assays prior to treatment, so each disc served as its own control. Afterwards, HA discs were exposed to the chosen treatment, reattached to the glass specimen and post-treatment measurements of dissolution rate were made at 30, 60 and 90 minutes (totaling 3 consecutive periods of 30 minutes each). Fresh citric acid solutions were used for each 30-min assay.

The rate of dissolution was calculated as the slope of the linear portion from the graphic obtained of acid volume versus time ($mL.s^{-1}$). This was converted to nmol of HA $min^{-1}.mm^{-2}$ using the area of the HA disc and a pH- and acid-dependent factor converting micromoles of acid to micromoles of hydroxyapatite.[26]

Deposition of salivary coating

Saliva was collected from two healthy volunteers participating in a saliva bank from University of Bristol. Volunteers were directed to decline the donation of saliva in the following situations: (1) they had recently taken any medication, (2) habitual smoker, (3) pregnant or (4) if

they had any upper respiratory tract infections in recent times. As required, each volunteer chewed a square of Parafilm to stimulate salivary flow and expectorated saliva into a tube until reach the 20-ml level of a polystyrene universal tube. These samples were pooled and centrifuged using a Centaur 1 (MSE, London, UK) at 4000 g for 15 min at ambient temperature. The supernatant was immediately used to treat HA specimens (2mL/ HA disc).

Treatment

Phase 1 (Native discs). After determining the mean baseline dissolution rate, HA discs (n=72) were divided into 9 groups (n=8) and treated for 2 min by immersion (with gentle stirring) in the following solutions: 1100 µg F/mL (1100); 1% or 8% HMP (1%HMP; 8%HMP); 1% or 8% TMP (1%TMP; 8%TMP); 1,100 µg F/mL with 1% HMP (1100 1%HMP); 1100 µg F/mL with 8% HMP (1100 8%HMP); 1100 µg F/mL with 1% TMP (1100 1%TMP) and 1100 µg F/mL with 8% TMP (1100 8%HMP). After rinsing in deionised water, three measurements of post-treatment were performed at 30, 60 and 90 min by the same way those baseline measurements.

Phase 2 (Saliva coated discs). For the saliva-coated HA discs, restriction in the number of experimental groups was necessary due to the nature of the experiment regarding the saliva amount. These groups chosen were based on those that showed the best overall results in phase one. Four groups were chosen from the non-saliva-treated discs to observe the influence of salivary coating with phosphates and fluoride. A group treated with deionised water (DIW) was included as a control to observe the effect of saliva alone. Groups with phosphates that led to higher reduction of dissolution rate were selected as following: 1100 1% HMP; 1100 8% HMP; 1100 1% TMP; and 1100 8% TMP. After obtaining the control measurements of dissolution rate, discs (n=40) were immersed in pooled saliva supernatant for 2h and incubated at 37°C. After this, the discs were stored in a damp environment until the treatment with the solutions selected. HA discs were therefore treated with the selected groups by the same way for non-saliva discs. Finally, the post-treatment dissolution rate was measured on each disc at three post-treatment times, as described above.

Statistical Analysis

For statistical analysis, SigmaPlot 12.0 software was used and the significance limit was set at 5%. Data from non-saliva and saliva coated discs exhibited a normal distribution (determined using the Shapiro-Wilks test). Treatment solutions, time (baseline control, post-treatment 1, 2 and 3) and presence or absence of saliva were considered as variation factors. A control value was determined for each specimen by averaging the three control runs before treatment. Then, data of groups from non-saliva coated discs were submitted to 2-way ANOVA,

followed by Fisher test. When saliva was considered as variable, the 3-way ANOVA was employed and the post hoc test used was Holm-Sidak test.

Results

In phase 1, exposure to 1100 µg F/mL promoted a significant reduction in the dissolution rate (12%) when compared with baseline values ($p=0.041$) in the first post-treatment run. This reduction decreased beyond the first 30 min and did not persist over time (Figure1).

Exposure to TMP at concentrations of 1 and 8% exhibited a similar profile of dissolution rate (Figure 2a and 2b) and reduced dissolution rate at 9% ($p=0.009$) and 13% ($p=0.016$), respectively, in the first post treatment run. This reduction persisted over time for the 8%TMP, presenting 10% of reduction in the third post-treatment run ($p=0.048$). When either 1 or 8% TMP concentrations were associated to fluoride (1100 µg F/mL), a significant reduction in HA dissolution was seen for 1100 8%TMP over time, being 22% lower than the baseline values ($p<0.001$) in the first post-treatment run and 8% ($p=0.048$) in the third post-treatment run (Figure 2c and 2d). The maximum reduction of dissolution rate in this study was observed for HMP. When 1 and 8% of HMP were assessed, the reduction of dissolution rate was, respectively, 24% ($p<0.001$) and 61% ($p<0.001$) at the first post-treatment run, 17% ($p=0.004$) and 41% ($p<0.001$) at the second post-treatment run, and 4% ($p=0.374$) and 40% ($p<0.001$) at the third post-treatment, showing a significant reduction of dissolution rate versus baseline over time for HMP at 8% (Figure 3a and 3b). For the 1100 1%HMP and 1100 8%HMP, the reduction rates were very similar, but persisted higher for the 1100 8% HMP in the second post-treatment ($p<0.001$) (Figure 3c and 3d). A comparison among groups can be observed in Figure 4.

Figure 5 shows the results from the saliva coated discs regarding the percentage reduction in the dissolution rate. There was a significant reduction of dissolution rate ($p<0.001$) for all groups evaluated in the presence of salivary coating, when compared with their treatment with DIW or with their counterparts not covered by salivary coating. Also, the presence salivary coating alone resulted in a significant reduction of the hydroxyapatite dissolution rate compared to the baseline rate ($p<0.001$), as seen for discs treated with DIW. The higher reduction of dissolution rate and persistence of effect over time in the presence of saliva was observed when HMP (1 and 8%) was associated with fluoride. There was a higher persistence of effect over time for the 1100 associated with 8% HMP ($p<0.001$).

Discussion

TMP and HMP associated with fluoride have been shown effective for both dental caries and erosion.[4,5,8,10-17] As pH-stat method has been used successfully as a technique to study dental erosion, providing information about interactions with hydroxyapatite and the persistence

of the inhibitory effect during repeated erosive challenges, in this study this method was employed to investigate the effect of these phosphates with or without fluoride.

In the present study, both phosphate salts evaluated inhibited dissolution of HA by different concentrations. The reduction of dissolution rate of HA treated with fluoride did not persist significantly further than the first post-treatment run, which is in agreement with Jones et al.[25] Although the fluoride concentration used in the present study was higher (1100 µg F/mL) than that used by Jones et al.,[25] (300 µg F/mL), no additional effect on HA dissolution was observed. The protective effect of fluoride against dental erosion is probably given by a formation of a protective layer that containing calcium fluoride (CaF_2),[2] and the adsorbed fluoride could be reduced by the acid challenge over time.[25]

Regarding the polyphosphates evaluated, higher inhibitory effect was found when compared with 1100, especially for HMP. For TMP alone, a similar dissolution profile was observed for both concentrations evaluated, showing a sustained inhibition over time for the 8% TMP. When TMP was associated with fluoride, a higher immediate and a sustained effect for the 1100 8% TMP was observed (Figure 2a and 2b). Based on the current results [13,14] and on previous studies, it becomes clear that an appropriate F/TMP ratio can reduce enamel demineralisation as well as the mineral loss in erosive challenges. Such effects, however, are not associated with formation of high amounts of CaF_2, as described for fluoride. This effect seems to be related to a formation of a "barrier" of TMP on the enamel surface, which limits the diffusion of acid into the enamel as well as the deposition of CaF_2 on this barrier, and the retention of ions (Ca^{++} and CaF^{+}) in the TMP molecule, which would be released during pH challenges.[13,28] TMP in the absence of fluoride has shown a little or no action on dental enamel.[4]

As has been stated for TMP, HMP has been shown to provide a less soluble hydroxyapatite as well as reduce the ion diffusion into the enamel.[19] Camara et al.[8] suggested that HMP is capable of binding to surface enamel and remain bound and also demonstrated that, in the absence of fluoride, a significant reduction of mineral loss was observed 0.5% HMP was used, when compared to placebo, suggesting that HMP (negatively charged) can adsorb at the positive sites of enamel surfaces forming a “coat” after treatment that acts as a protective layer on the enamel. In the present study a higher reduction of dissolution rate was found for the 8% HMP concentration (with or without fluoride), which differ that data found by Camara et al.[8] that showed higher mineral loss with increased HMP concentration in association with fluoride (250 µg F/mL) when an *in vitro* caries model was used. In the present study HA dissolution was assessed using an erosion-like model with a different F concentration, which might have influenced in the different results obtained. Regarding HMP and TMP structure, these salts have six and three phosphate groups, respectively, what might help explain the greater results obtained with HMP; when in the oral environment, HMP could form a stronger “barrier” on

enamel surface and provide a higher amount of binding sites for the retention of ions (Ca^{++} and CaF^{+}) in comparison with TMP.

Considering the influence of saliva on the effect of anti-erosive agents, the presence of a salivary coating was considered in the second set of experiments. An exposure time of 2h to saliva ensured an effect on HA discs and allowed to investigate the interactions with polyphosphates in this study. According to literature a significant effect can be obtained for exposure times $\geq$ 60min.[25,29] As expected, the formation of a salivary coating resulted in a significant reduction of the hydroxyapatite dissolution rate (33%), which is in line with a previous study conducted with the same methods of analysis,[25] showing a reduction in HA dissolution of 41%. Furthermore, the presence of salivary coating led to higher protection for all groups when compared to discs without pellicle (Figure 5). The profile obtained was similar to those obtained for native discs; however all tested solutions with fluoride and associated to HMP or TMP promoted an additional effect in the presence of saliva and polyphosphates. Nonetheless this effect (immediate and sustained) was greater for the solutions containing HMP, showing that the salivary coating did not reduce or hinder the polyphosphates action.

To conclude, confirming this study hypothesis, the reduction of HA dissolution rate as well as the duration of this effect was influenced by fluoride, type and concentration of phosphate salt and enhanced by the presence of a salivary coating. This is important from a clinical point of view, since the tooth surfaces *in vivo* are coated with salivary coating under normal conditions.

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Figure Legends

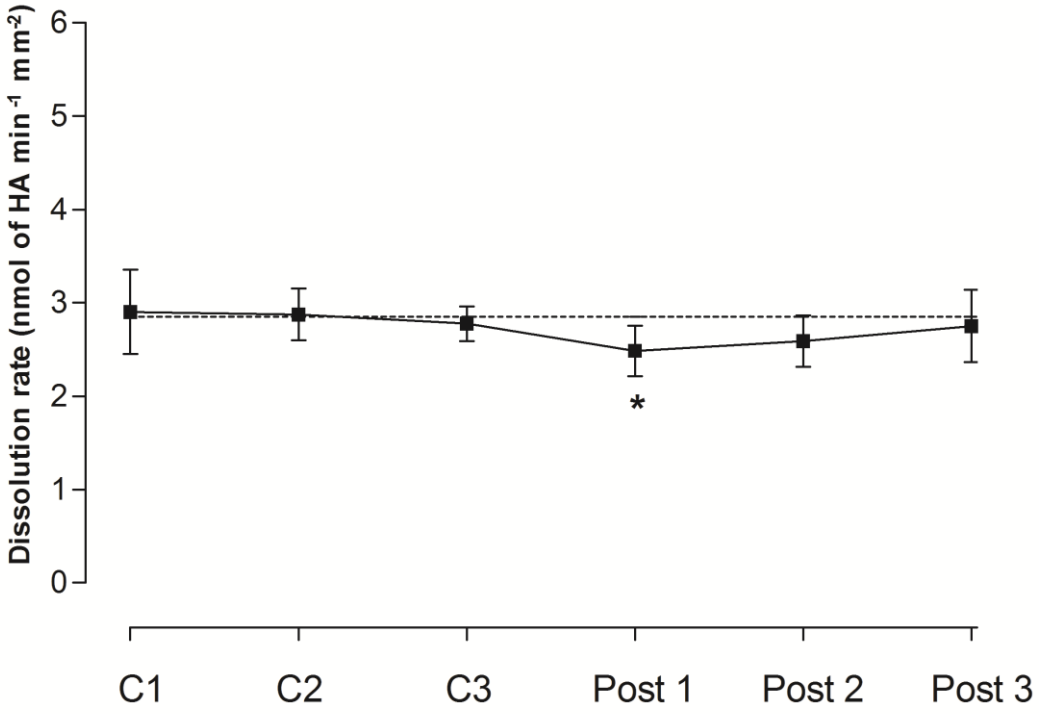
Figure 1. Dissolution rate of HA for the three control runs before treatment (C1-3) and after treatment (Post 1-3) with 1100 µg F/mL. Dashed line= mean control rate. *Significantly different from mean baseline control.

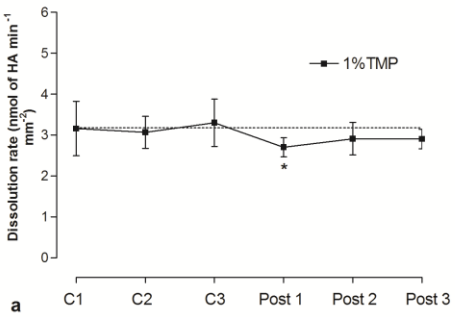
Figure 2. Dissolution rate of HA for the three control runs before treatment (C1-3) and after treatment (Post 1-3) with TMP and fluoride. Dashed line= mean control rate. *Significantly different from mean baseline control.

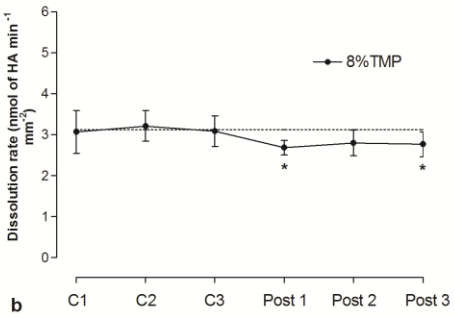
Figure 3. Dissolution rate of HA for the three control runs before treatment (C1-3) and after treatment (Post 1-3) with HMP and fluoride. Dashed line= mean control rate. *Significantly different from mean baseline control.

Figure 4. Graphic representation of HA dissolution rate reduction according to the treatments, and post-treatment run. a= post-treatment 1 (30min), b=post-treatment 2 (60min) and c= post-treatment 3 (90min). Bars indicate the standard deviation of the mean. Different lowercase letters represent statistical difference between the groups (Fisher test, $p < 0.001$). * indicate significant difference from mean baseline control.

Figure 5. Graphic representation of HA dissolution rate reduction according to the treatments, saliva and post-treatment run. a= post-treatment 1 (30min), b=post-treatment 2 (60min) and c= post-treatment 3 (90min). Bars indicate the standard deviation of the mean. Distinct capital letters represent statistical difference between the groups without salivary coating. Different lowercase letters represent statistical difference between the groups with salivary coating (Holm-Sidak, $p < 0.001$).


Dissolution rate (nmol of HA min-1 mm-2)
6
5
4
3
2
1
0
*
C1
C2
C3
Post 1
Post 2
Post 3


Dissolution rate (nmol of HA min -1 mm -2)
1%TMP
C1
C2
C3
Post 1
Post 2
Post 3
a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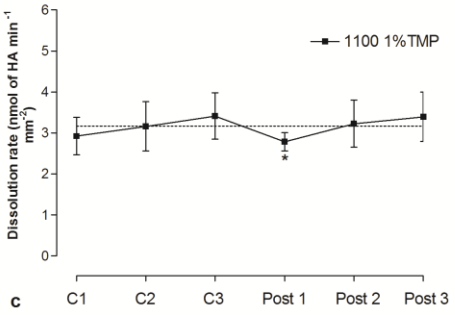
8%TMP
C1
C2
C3
Post 1
Post 2
Post 3
b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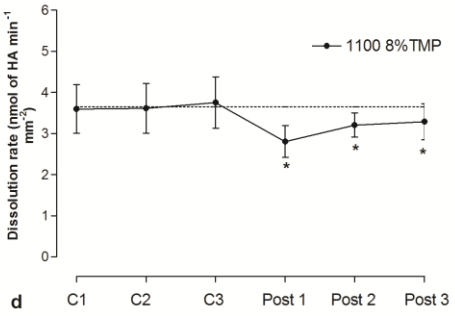
C1
C2
C3
Post 1
Post 2
Post 3
c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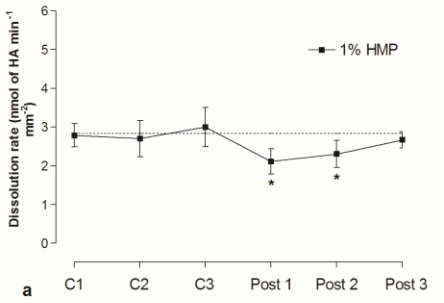
C1
C2
C3
Post 1
Post 2
Post 3
d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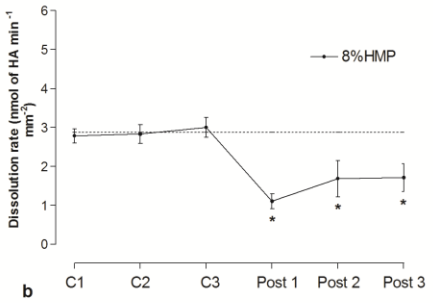
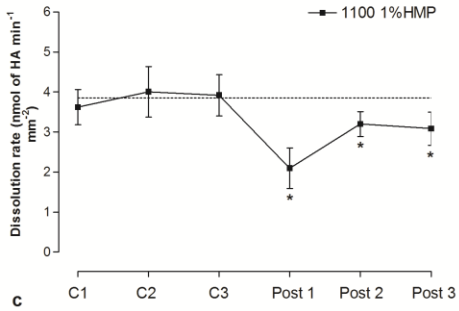
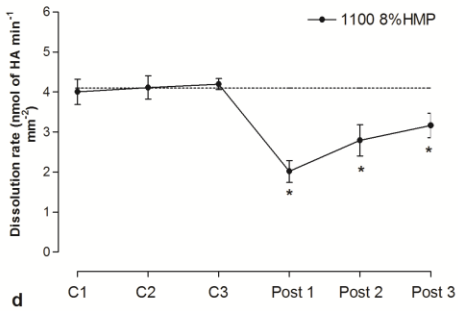
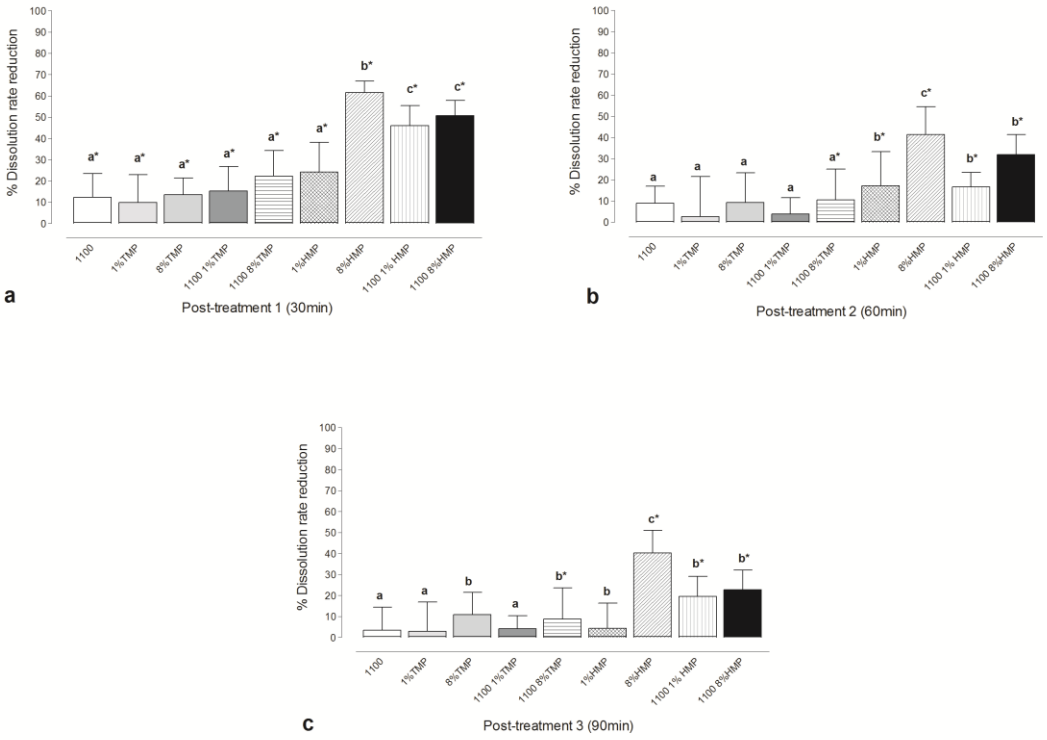
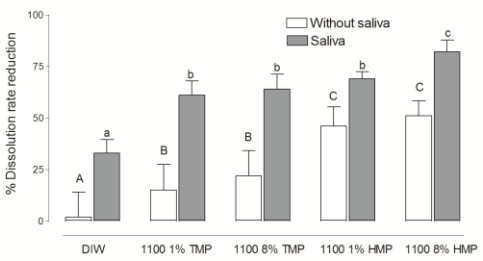
1% HMP
C1
C2
C3
Post 1
Post 2
Post 3
a


Dissolution rate (nmol of HA min-1 mm-2)
8%HMP
C1
C2
C3
Post 1
Post 2
Post 3
b


Dissolution rate (nmol of HA min-1 mm-2)
1100 1%HMP
C1
C2
C3
Post 1
Post 2
Post 3
c

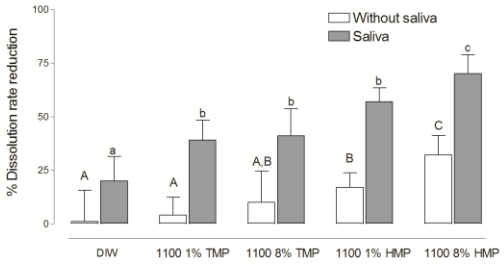

Dissolution rate (nmol of HA min-1 mm-2)
1100 8%HMP
C1
C2
C3
Post 1
Post 2
Post 3
d


% Dissolution rate reduction
100
90
80
70
60
50
40
30
20
10
0
a*
a*
a*
a*
a*
a*
b*
c*
c*
1100
1%TMP
8%TMP
1100 1%TMP
1100 8%TMP
1%HMP
8%HMP
1100 1% HMP
1100 8%HMP
a
Post-treatment 1 (30min)
% Dissolution rate reduction
a
a
a
a
a*
b*
c*
b*
b*
b
Post-treatment 2 (60min)
% Dissolution rate reduction
a
a
b
a
b*
b
c*
b*
b*
c
Post-treatment 3 (90min)



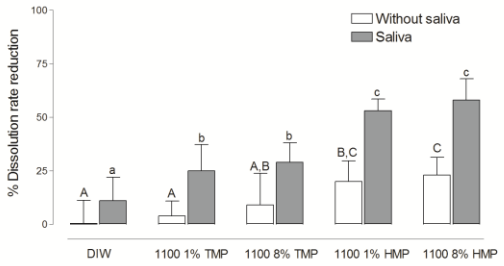
a

Post-treatment 1 (30min)



b

Post-treatment 2 (60min)



c

Post-treatment 3 (90min)